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Characterization of Acrolein-Glycerophosphoethanolamine Lipid Adducts Using Electrospray Mass Spectrometry

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Abstract

Acrolein is a toxic, highly reactive α,β -unsaturated aldehyde. In the current study, the products of acrolein after reaction with glycerophosphoethanolamine (GPEtn) lipids have been characterized using electrospray tandem mass spectrometry. The major product formed involves the addition of two acrolein molecules to the primary amine of GPEtn lipids and subsequent aldol condensation to form 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-formyl-4-hydroxy)piperidine (FHP) lipids. Upon sodium borohydride reduction, 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-4-hydroxy)piperidine (HMHP) lipids and 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-3,4-dehydro)piperidine (HMDP) lipids were selectively detected using electrospray tandem mass spectrometry by employing precursors of m/z 256.1 and 238.1 scans, respectively. HMHP lipid and HMDP lipid molecular species were detected upon treatment of HL-60 cells with concentrations of acrolein as low as 10 μ M. While the biological implications of these acrolein GPEtn adducts have yet to be established, these structural characterization studies reported herein reveal the facile formation of acrolein GPEtn lipid adducts in vitro, which could influence subsequent biochemical events within the cell.

Introduction

Acrolein is a volatile, highly reactive α,β -unsaturated aldehyde that is produced by many mechanisms relevant to human exposure (1,2). Acrolein is also one of the most abundant volatile, electrophilic compounds present in cigarette smoke, and concentrations of acrolein in the pulmonary lining fluid of human subjects smoking cigarettes have been estimated to be as high as 80 μ M (3). In addition, the concentration of acrolein in the saliva of smokers has been reported to range from 5 to 38 μ M (4). Acrolein also is known to elicit several different biochemical responses when exposed to cells from transcription factor activation (5) to cell death (6,7). The exact mechanism of these cellular effects of acrolein has not been established, but there is evidence that the alteration of these biochemical processes is due to the high chemical reactivity of acrolein and resultant covalent binding to nucleophilic molecules in the cell.

Among all α,β -unsaturated aldehydes, acrolein is one of the strongest electrophiles and has exhibited reactivity with cellular nucleophiles, such as DNA, proteins, and glutathione (8). There are two main reactions that can occur with α,β -unsaturated aldehydes and molecules that contain primary amino groups. The first is Schiff base formation, which occurs by the condensation of an amine with the aldehyde through a carbinolamine intermediate to form R-CH₂=N-R'. The second reaction between α,β -unsaturated aldehydes and amines is a Michael addition, which involves the 1,4-addition of a primary amine to the α,β -unsaturated carbonyl

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compound to form a β -substituted propanal [R'NH-CH(R)-CH-CHO]. Uchida and co-workers have shown that acrolein modification of proteins involves sequential addition of two acrolein molecules to a lysine residue by 1,4-Michael addition, followed by aldol condensation and dehydration to form a six-membered ring resulting in N^ε-(3-formyl-3,4-dehydropiperidino)lysine (9). Another acrolein-lysine adduct has been more recently identified as N^ε-(3-methylpyridinium)lysine, which involved intermediate formation of a Schiff base (10). This Schiff base intermediate further reacted with a second acrolein molecule by a 1,4-Michael addition to generate an imine derivative that converted into the final lysine adduct. Antibodies have been raised against these acrolein-lysine adducts and have revealed elevated levels of acrolein-modified proteins in tissues of several degenerative diseases such as atherosclerosis and Alzheimer's disease (11,12).

The phospholipid bilayer of cells serves as an initial cellular structure that reactive oxygen species must cross prior to encountering nucleophilic targets within the cell. Two abundant glycerophospholipids, glycerophosphoethanolamine (GPEtn)¹ and glycerophosphoserine (GPSer) lipids, found in the cell membrane, contain a primary amine group. While it has been previously established that GPEtn and GPSer lipids react with aldehydes (13–16), there have not been studies reporting the reaction of either of these aminophospholipids with acrolein. Furthermore, most of these previous aminophospholipid/aldehyde adduct studies have focused primarily on the formation of aldehyde-modified GPEtn and GPSer products in solution, and few studies have shown that these covalently modified GPEtn and GPSer species are present in cell membranes (17,18).

In the current study, acrolein was reacted with 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1a/18:1-GPEtn),² and the structures of the major products were characterized by electrospray mass spectrometry and ancillary techniques. The acrolein-modified 18:1a/18:1-GPEtn standard was employed to determine chromatographic and mass spectrometric properties and establish a sensitive assay for such adducts. Additionally, the presence of acrolein-modified GPEtn lipids was established after exposure of HL-60 cells to acrolein. Acrolein adducted to GPEtn lipids was found to be formed in a dose-dependent manner at a concentration of acrolein consistent with a role of this reaction pathway, mediating various disease states associated with reactive oxygen species and cigarette smoke exposure.

Experimental Procedures

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1a/18:1-GPEtn) and 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (14:0a/14:0-GPEtn) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Hanks' balanced salt solution (1×) without calcium and magnesium (HBSS[−]) was obtained from Invitrogen (Carlsbad, CA). [1,1,1-D₃]methanol was

¹Abbreviations: BSTFA, bis(trimethylsilyl)trifluoroacetamide; MOX, methoxyamine hydrochloride; HPLC, high-performance liquid chromatography; GPEtn, glycerophosphoethanolamine lipid; GPSer, glycerophosphoserine lipid; 18:1a/18:1-GPEtn, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; 18:1a/18:1-FHP, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-formyl-4-hydroxy)piperidine; 18:1a/18:1-FDP, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-formyl-3,4-dehydro)piperidine; 18:1a/18:1-HMHP, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-4-hydroxy)piperidine; 18:1a/18:1-HMDP, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-3,4-dehydro)piperidine; FHP lipid, 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-formyl-4-hydroxy)piperidine; FDP lipid, 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-formyl-3,4-dehydro)piperidine; HMHP lipid, 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-4-hydroxy)piperidine; HMDP lipid, 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-3,4-dehydro)piperidine; 2,4-DNPH, 2,4-dinitrophenylhydrazine; HBSS[−], Hanks' balanced salt solution without calcium and magnesium.

²Abbreviations for individual GPEtn molecular species used in this paper are as follows: n:j/k/s:t-GPEtn (e.g., 16:0p/20:4-GPEtn), where n is the number of carbon atoms in the *sn*-1 substituent and j is the number of double bonds in the *sn*-1 hydrocarbon chain; k represents the type of *sn*-1 linkage, where p refers to plasmalogen (1-O-alk-1'-enyl), e refers to ether (1-O-alkyl), and a refers to acyl; s is the number of carbons, and t is the number of double bonds in the *sn*-2 substituent.

purchased from Cambridge Isotopes Laboratories, Inc. (Andover, MA). Acrolein, 2,4-dinitrophenylhydrazine (2,4-DNPH), methoxyamine hydrochloride (MOX), and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma (St. Louis, MO). Sodium borohydride was purchased from Aldrich (Milwaukee, WI). HPLC solvents and 1 N sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ) and used for HPLC and derivatization protocols. Acrolein 2,4-DNPH standard was purchased from Supelco (Bellefonte, PA).

Synthesis of Acrolein-Modified 18:1a/18:1-GPEtn Standard and Acrolein-Modified 14:0a/14:0-GPEtn Internal Standard

18:1a/18:1-GPEtn (700 nmol) was incubated with acrolein (1.4 μ mol) in methanol/methylene chloride/HBSS⁼ (2/1/0.8) (500 μ L) at 37 °C for 3 h. The acrolein stock solutions were in ethanol and made fresh daily. The acrolein-modified 18:1a/18:1-GPEtn product was then extracted by the addition of methylene chloride–methanol–water according to a modified method of Bligh and Dyer (19), substituting methylene chloride for chloroform. The standard was dried down under a stream of nitrogen and resuspended in ethanol (1 mL). Aliquots were then taken for reduction, derivatization, and mass spectrometric analysis.

The acrolein-modified GPEtn internal standard used in these experiments was synthesized according to the above protocol using 14:0a/14:0-GPEtn (700 nmol) as a starting material. The resulting products were reduced using sodium borohydride according to the procedure described below. The internal standard was stored in ethanol (2 mL), and 500 ng aliquots were added to the samples during the Bligh–Dyer extractions.

Reduction and Derivatization of Acrolein–GPEtn Products

To aid in structural elucidation of the products formed upon reaction of acrolein and glycerophosphoethanolamine lipids, both reduction and derivatization procedures were performed on the acrolein-modified 18:1a/18:1-GPEtn standard. Any aldehyde or ketone groups present on the acrolein-modified 18:1a/18:1-GPEtn standard were reduced by the addition of 1 M sodium borohydride in ethanol (100 μ L) to the acrolein-modified 18:1a/18:1-GPEtn standard (1 μ g) in methanol/methylene chloride/HBSS⁼ (2/1/0.8) (0.5 mL) and incubated at 4 °C for 1 h. The reduced acrolein-modified 18:1a/18:1-GPEtn standard was extracted using the modified Bligh and Dyer extraction, and the methylene chloride layer was taken to dryness under a stream of nitrogen and resuspended in ethanol (500 μ L). To provide more structural information, the functional groups present on the unreduced acrolein-modified 18:1a/18:1-GPEtn standard were derivatized with MOX and BSTFA. The aldehyde or ketone functional groups present on the acrolein-modified 18:1a/18:1-GPEtn standard were derivatized by MOX using a gas phase procedure (20). Briefly, 1 N sodium hydroxide (1 mL) and MOX (83 mg) were added together and attached to an enclosed glass apparatus. During a 30 min incubation, the liberated CH₃ONH₂ gas derivatized the aldehyde or ketone groups present on the acrolein-modified 18:1a/18:1-GPEtn standard. After MOX derivatization, the hydroxyl groups present on the acrolein-modified 18:1a/18:1-GPEtn standard were derivatized using BSTFA (50 μ L) and acetonitrile (50 μ L) for 1 h at 60 °C. After derivatization, the samples were dried down under a stream of nitrogen and resuspended in ethanol (500 μ L). Aliquots of the reduced and derivatized acrolein-modified 18:1a/18:1-GPEtn standard were then taken for mass spectrometric analysis.

Electrospray Ionization Mass Spectrometry

The acrolein-modified 18:1a/18:1-GPEtn standard, along with the reduced and derivatized acrolein-modified 18:1a/18:1-GPEtn standard, were infused into a Sciex API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, Canada) at a flow rate of 10 μ L/min. The concentration of the standards that was infused in these experiments was approximately 500

nM in methanol:acetonitrile:water (60:20:20) with 1 mM ammonium acetate. The relevant experimental parameters in the positive ion mode for both full scan and collision-induced dissociation experiments were an electrospray voltage of 5000 V, a declustering potential of 50 V, and a focusing potential of 350 V. The collision-induced dissociation mass spectra of the reduced acrolein-modified 18:1a/18:1-GPEtn products were acquired in the positive ion mode at 3 s/scan over the mass range m/z 50–900 with the collisional offset reported in the figure captions. The collisional offset used in the precursors of m/z 256.1 and m/z 238.1 scans for the detection of reduced acrolein-modified GPEtn products in HL-60 cells was 40 V.

HL-60 Cells

Human promyelocytic leukemia cell line HL-60 was obtained from ATCC (Manassas, VA). These HL-60 cells were maintained using RPMI 1640 (1×) (Mediatech, Inc., Herndon, VA) supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT) and 1% penicillin/streptomycin (Mediatech, Inc.) at 37 °C in a 5% CO₂-humidified incubator. The cells were cultured every 3 days to a cell density of 0.3×10^6 cells/mL.

Acrolein Experiments with HL-60 Cells

The HL-60 cells were spun down, rinsed with HBSS[−], and then resuspended in HBSS[−] at a concentration of 10×10^6 cells/mL. The cell suspension was then aliquotted into separate tubes for the control and acrolein-treated samples so that each experimental condition used a total of 40×10^6 HL-60 cells. Acrolein was then added to the HL-60 cell suspensions to a final concentration of 10–500 μ M. The acrolein stock solutions were in ethanol, and these solutions were made fresh daily for each set of experiments. The final percent of ethanol present in the HL-60 suspension was limited to lower than 0.1%. Experiments were also performed where acrolein solutions were made in HBSS[−] instead of ethanol. The results reported in this paper did not change when HBSS[−], rather than ethanol, was used to introduce acrolein into the system. The samples were allowed to incubate at 37 °C for 30 min, and the cell membranes were pelleted by centrifugation. The supernatant with any excess acrolein was removed, and the cell pellet was rinsed with HBSS[−]. The cell pellet was resuspended in methanol/methylene chloride/HBSS[−] (2/1/0.8) (0.5 mL), the internal standard was added, and reduction by sodium borohydride was achieved as described above. After sodium borohydride reduction, the phospholipids were extracted as described above.

Normal and Reversed Phase Chromatography

After sodium borohydride reduction, the different lipid classes present in HL-60 cells were separated using normal phase high-performance liquid chromatography (HPLC) with an Ultremex 5 μ Si (4.6 mm $\times$ 250 mm) column (Phenomenex, Torrance, CA). The normal phase solvents used for the separation of phospholipid classes were 30:40 hexane/2-propanol (solvent A) and 30:40:7 hexane/2-propanol/water with a final concentration of 1 mM ammonium acetate (solvent B). The initial mobile phase was 47% solvent B at a flow rate of 1 mL/min. This initial mobile phase was held for 6 min, and then, a linear gradient was started to 100% solvent B in 20 min. This was followed by isocratic elution at 100% solvent B for 14 min. Detection of the reduced acrolein–GPEtn adducts was achieved using a positive ion Q1 scan. A postcolumn split was used to yield 50 μ L/min into the mass spectrometer and 950 μ L/min to a fraction collector, which collected one fraction per min. The reduced acrolein-modified GPEtn adducts eluted from the normal phase HPLC column under these conditions at 14–20 min, and these normal phase fractions were separated according to lipophilicity by reversed phase HPLC with a Columbus 5 μ m C₁₈ (2.0 mm $\times$ 150 mm) column (Phenomenex). The reversed phase solvents used for the separation of reduced acrolein-modified GPEtn products according to lipophilicity were 60:20:20 methanol/acetonitrile/water with 1 mM ammonium acetate (solvent A) and 1 mM methanolic ammonium acetate (solvent B). The initial mobile phase was 0% solvent B at

a flow rate of 0.2 mL/min. This initial mobile phase was held for 2 min, and then, a linear gradient was started to 100% solvent B in 18 min. This was followed by isocratic elution at 100% solvent B for 20 min. The reduced acrolein-modified GPEtn phospholipids were detected during the reversed phase chromatographic separation by using precursors of m/z 256.1 and m/z 238.1 scans. Turbo ion spray was used in this particular experiment with the turbo heater temperature at 350 °C and the drying gas delivered at 250 mL/min.

Cell Death

To assess the toxicity of different acrolein concentrations, cell death was determined by exclusion of trypan blue as previously described using the manufacturer's protocol (Sigma Aldrich). Cell death was determined following exposure for 5 min to 0.4% trypan blue solution prepared in HBSS⁺.

Preparation and Analysis of 2,4-DNPH Derivatives

The cell suspensions for this set of experiments were prepared as described above, and the final concentration of acrolein used was 10, 30, 70, and 100 μ M, and the incubation time used prior to the addition of 2,4-DNPH was 0, 1, 3, 10, and 30 min. Derivatization of acrolein with 2,4-DNPH to form the corresponding hydrazone was achieved as previously described (21). Briefly, a 12 mM solution of 2,4-DNPH (50 mg) in acetonitrile with 2% formic acid (100 μ L) was mixed with an HL-60 cell suspension containing acrolein (100 μ L) and incubated at room temperature for 1 h. After the hour long incubation, the sample was centrifuged to pellet debris and transferred to an autosampler vial. The derivatized acrolein samples (100 μ L) were injected onto a Columbus 5 μ m C₁₈ (2.0 mm $\times$ 150 mm) column (Phenomenex), and separation was achieved using water (solvent A) and acetonitrile (solvent B) as the HPLC solvents. The initial mobile phase was 40% solvent B at a flow rate of 0.2 mL/min. This initial mobile phase was held for 3 min, and a linear gradient was started to 70% solvent B in 12 min and then finally to 100% solvent B in 1 min. This was followed by isocratic elution at 100% solvent B for 2 min. The derivatized acrolein hydrazone products were detected by monitoring between 300 and 420 nm using a Shimadzu diode array detector (SPD-M10AVP). The acrolein hydrazones eluted from the reversed phase column under these conditions at 9.1 min and the maximum UV absorbance occurred at 372 nm. Acrolein-2,4-DNPH standard (Supelco) was used to establish the standard curve.

Results

The major products formed after the incubation of 18:1a/18:1-GPEtn with acrolein were assessed by mass spectrometric analysis in the positive ion mode. Abundant $[M + H]^+$, $[M + Na]^+$, and $[M - H + 2Na]^+$ ions were observed in the positive ion mass spectrum before reaction of 18:1a/18:1-GPEtn with acrolein at m/z 744.5, 766.5, and 788.5, respectively (Figure 1a). After the reaction of 18:1a/18:1-GPEtn with 3 mM acrolein, the abundant $[M + H]^+$, $[M + Na]^+$, and $[M - H + 2Na]^+$ ions shifted to m/z 888.6, 910.6, and 932.6 (Figure 1b), which corresponded to the addition of 145 Da onto the neutral 18:1a/18:1-GPEtn starting material. Some of the possible products of the reaction of GPEtn lipids with acrolein are shown in Scheme 1. A Schiff base ($CH_2=CH-CH_2=N-R$) adduct (**I**) would have a $[M + H]^+$ at m/z 782.5, while a 1,4-Michael addition $[R-NH-(CH_2)_2-CHO]$ product (**II**) would generate a $[M + H]^+$ at m/z 800.5. However, neither of these ions was observed in the positive ion mass spectrum (Figure 1b) of the reaction products of acrolein with 18:1a/18:1-GPEtn. Previously, it was determined that one of the major products formed upon the reaction of primary amino groups on peptides with acrolein resulted from the 1,4-Michael addition of two acrolein molecules and subsequent aldol condensation followed by dehydration (14). An analogous aldol condensation product (**IV**) from our 18:1a/18:1-GPEtn standard would be expected to have a

$[M + H]^+$ at m/z 856.5. This ion was present in the positive ion mass spectrum (Figure 1b), but it was a fairly minor product.

Because the aldol condensation product retained an aldehyde moiety and the electrospray solvent contained methanol, it was thought that the major ion at m/z 888.6 in Figure 1b could be due to the methanol hemiacetal of the aldol condensation product. To probe this hypothesis, ethanol and $[CD_3]$ methanol were used as the electrospray solvents. The abundant $[M + H]^+$ at m/z 888.6 in the positive ion mass spectrum was shifted by 14 amu to m/z 902.5 when electrosprayed in ethanol and by 3 amu to m/z 891.5 when electrosprayed in $[CD_3]$ methanol (data not shown). From this data, the most abundant $[M + H]^+$ at m/z 888.6 in the positive ion mass spectrum (Figure 1b) was consistent with the methanol hemiacetal of the aldol condensation product from two acrolein molecules adducted to 18:1a/18:1-GPEtn. Additionally, the m/z at 910.6 and 932.6 would correspond to be the $[M + Na]^+$ and the $[M - H + 2Na]^+$ of the methanol hemiacetal. This methanol hemiacetal of the aldol condensation product from two acrolein molecules adducted to 18:1a/18:1-GPEtn (**IV**) only forms under electrospray conditions. Hemiacetal formation from aldehydes is typically an acid-catalyzed reaction, and the only time that this product is introduced into an acidic system is during electrospray mass spectrometry analysis. The water used in the electrospray solvent system has a pH of 4.8. At all other times during the course of the experiments, HBSS⁺ was used as the aqueous solvent with pH 7.4. Therefore, it is thought that the major product is present in pure organic solution, and in pH 7.4, HBSS⁺ is present as an aldehyde and not as a hemiacetal.

The adducts of 18:1a/18:1-GPEtn with acrolein reported above were formed during the incubation of 18:1a/18:1-GPEtn with a molar excess of acrolein, and it was thought that this major product might be a result of the excess acrolein added to the reaction mixture. Additionally, the concentration of acrolein found in respiratory tract lining fluids after exposure to cigarette smoke has been estimated to be as high as 80 μ M (3), and most likely, acrolein produced in vivo from lipid peroxidation and other endogenous sources would not reach millimolar levels. Therefore, the products formed upon the reaction of 18:1a/18:1-GPEtn (1.5 mM) with 10–500 μ M acrolein, which might be a more relevant mole ratio of reactants, were examined. Even at these lower relative concentrations of acrolein (10, 100, and 500 μ M), the methanol hemiacetal product ion at m/z 888.6 was observed, although at lower abundance (Figure 2). There were no product ions present at m/z 800.6, which corresponded to the 1,4-Michael addition of one acrolein molecule to 18:1a/18:1-GPEtn (**II**). This suggested that the aldol condensation product, which involves the addition of two acrolein molecules, was the major product even when the ratio of acrolein to aminophospholipid was less than one.

To verify that the major product formed upon reaction of acrolein with 18:1a/18:1-GPEtn was the aldol condensation product (**IV**), two derivatization studies were performed. The first derivatization procedure used was a MOX derivatization. Exposure of the acrolein 18:1a/18:1-GPEtn adduct to MOX resulted in an observed mass shift of the suspected aldol condensation product $[M + H]^+$ at m/z 856.8 to m/z 885.6, consistent with the addition of 29 u and conversion of the terminal aldehyde to a methoxime derivative (Figure 3a). The additional ions observed at m/z 907.6 and 929.6 were consistent with the $[M + Na]^+$ and $[M - H + 2Na]^+$ of the methoxime derivative of the aldol condensation product and with the acrolein 18:1a/18:1-GPEtn adduct retaining only one aldehydic group following aldol condensation. A second derivatization procedure with a trimethylsilylation reagent would convert hydroxyl groups to trimethylsilyl ethers with concomitant shift in observed mass by 72 u for each hydroxyl group present on the molecule. The methoxime derivative of the adduct (m/z 885.6) was shifted to an observed $[M + H]^+$ to m/z 957.6 (Figure 3b), which corresponded to the addition of a single trimethylsilyl group onto a hydroxyl group of the methoxime derivative of the acrolein 18:1a/18:1-GPEtn adduct. The additional ions present in the positive ion mass spectrum of the MOX/BSTFA derivatized acrolein adduct at m/z 979.6 and 1001.6 would correspond to the $[M +$

$\text{Na}]^+$ and $[\text{M} + 2\text{Na}]^+$ adduct. These data were consistent with the major product formed upon the incubation of acrolein with 18:1a/18:1-GPEtn being 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-formyl-4-hydroxy)piperidine (18:1a/18:1-FHP) (**IV**).

Because this adduct retained a reactive aldehydic group, it was decided to reduce the adduct to facilitate analysis of this compound in biological systems. The 18:1a/18:1-FHP was readily reduced by sodium borohydride (Figure 4a) to yield 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-4-hydroxy)piperidine (18:1a/18:1-HMHP) ($[\text{M} + \text{H}]^+$, m/z 858.6). The positive ion electrospray mass spectrum of the 18:1a/18:1-HMHP standard yielded an abundant $[\text{M} + \text{H}]^+$ at m/z 858.6 (Figure 4a), which corresponded to the addition of 2 amu onto the $[\text{M} + \text{H}]^+$ at m/z 856.6 of unreduced adduct (Figure 1b). The ions at m/z 880.7 and 902.6 corresponded to $[\text{M} + \text{Na}]^+$ and the $[\text{M} - \text{H} + 2\text{Na}]^+$ of the reduced 18:1a/18:1-GPEtn acrolein adduct. Collision-induced dissociation of the $[\text{M} + \text{H}]^+$ at m/z 858.6 yielded major product ions observed at m/z 603.5 and 256.1 (Figure 4b). The m/z 603.5 ion was also observed during the CID of 18:1a/18:1-GPEtn (22). This ion would be generated from cleavage of the phosphate–glycerol bond with a site of protonation at the *sn*-2 ester (23), which resulted in the neutral loss of (3-hydroxymethyl-4-hydroxy-piperidine)-phosphoethanol (255 amu). The product ion present at m/z 256.1 corresponded to cleavage of the phosphate glycerol with charge retention on the polar head group. A minor $[\text{M} + \text{H}]^+$ ion in the positive ion ESI mass spectrum after sodium borohydride reduction was observed at m/z 840.6 (Figure 4a), which was 18 amu less than the $[\text{M} + \text{H}]^+$ of 18:1a/18:1-HMHP. This product at m/z 840.6 was consistent with NaBH_4 -reduced 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-(3-formyl)-3,4-dehydropiperidine (**V**) and is referred to as 1,2-oleoyl-*sn*-glycero-3-phosphoethanol-(3-hydroxymethyl-3,4-dehydro)piperidine (18:1a/18:1-HMDP). CID was performed on this ion, and the major product ions observed were at m/z 603.5 and m/z 238.1 (Figure 4c). The product ion at m/z 603.5 was described above and is likely due to the neutral loss of (3-hydroxymethyl-3,4-dehydropiperidine)phosphoethanol (237 amu). The product ion present at m/z 238.1 corresponded to cleavage of the phosphorous–oxygen bond with charge retention on the polar head group of the acrolein-modified phospholipid. The product ions observed at m/z 256.1 and 238.1 were quite unique to acrolein-modified GPEtn lipids in that naturally occurring phospholipids do not generate these ions (24). Therefore, tandem mass spectrometry of the precursors of m/z 256.1 and 238.1 scans could be used to determine the presence of these acrolein-modified GPEtn lipids in biological samples.

To determine if acrolein GPEtn adducts were made in cells exposed to acrolein, HL-60 cells were incubated with 500 μM acrolein in HBSS[−] for 30 min. After 30 min, the cell membranes were pelleted and the supernatant retaining excess acrolein was removed. The cell pellet was resuspended in monophasic Bligh–Dyer conditions and reduced by sodium borohydride, and a Bligh–Dyer extraction was continued with phase separation (19). The reduced lipid extract was separated into phospholipid classes by normal phase HPLC using mass spectrometric detection (ESI positive ions) of the HPLC effluent. It was apparent that a new peak appeared between 14 and 20 min in the normal phase chromatogram of the acrolein-treated HL-60 cells as compared to control HL-60 cells (Figure 5a). This new HPLC peak, which eluted between GPEtn and GPSer, contained the acrolein-modified GPEtn species (see below). Additionally, the GPSer peak disappeared from the normal phase HPLC chromatogram after acrolein treatment. This phospholipid contained a primary amino group that was modified by acrolein. An acrolein GPSer adduct standard was synthesized and eluted in the GPCho fraction, but these particular adducts were not further examined.

The positive ion mass spectra of the normal phase fraction (8–13 min) indicated elution of GPEtn lipids present in HL-60 cells (Figure 5b), and the identity of the major molecular species labeled in the spectrum is indicated in Table 1. The identity of these molecular species was examined by CID studies of the corresponding $[\text{M} - \text{H}]^-$ to determine the fatty acyl groups

esterified to the glycerol backbone of phospholipids (24). The positive ESI mass spectrum of the acrolein-modified GPEtn lipid fraction present in HL-60 cells (14–20 min) indicated a family of acrolein-modified GPEtn lipids (Figure 5c). This summed mass spectrum was quite complicated due to the presence of $[M + H]^+$ from both the HMDP–lipid and the HMHP–lipid species.

To assign which of the positive ions (Figure 5c) corresponded to HMDP–lipid species and which corresponded to HMHP–lipid species, the normal phase fractions (14–20 min) were pooled and an aliquot was infused into a tandem quadrupole mass spectrometer. Precursor ion scanning for m/z 238.1 scan revealed those HMDP molecular species (Figure 6a) present in the pooled HPLC fraction. Further characterization of the phospholipids present in the pooled fractions was carried out by negative ion CID to determine fatty acyl substituents (Table 1). The precursor ions for m/z 238.1 appeared very similar to the distribution of positive ions in terms of relative abundance to that of GPEtn molecular species (Figure 5b) except that the ions from the acrolein-treated HL-60 cells were shifted by 96 amu. This increase of 96 amu as compared to the unmodified GPEtn lipids corresponded to the sodium borohydride-reduced product of the aldol condensation and subsequent dehydration of the 1,4-Michael addition of two acrolein molecules into GPEtn lipids (**V**). The structure is shown in Figure 4c. Additionally, the $[M + H]^+$ that was present in this precursor ion scan for m/z 238.1 was also observed in the positive ion scan of acrolein-modified GPEtn species (Figure 5c). The HMHP molecular species present in the Bligh–Dyer extract of acrolein-treated cells were detected using a precursor of m/z 256.1 scan (Figure 6b) and further characterized by negative ion CID as described above (Table 1). The abundance of these adducted molecular species (Figure 6b) was similar to the untreated HL-60 GPEtn molecular species (Figure 5b) but shifted by 114 amu. This increase of 114 amu as compared to unmodified GPEtn lipids corresponded to the sodium borohydride-reduced product of the aldol condensation of the 1,4-Michael addition of two acrolein molecules into GPEtn lipids (**IV**). This structure is shown in Figure 4b. The $[M + H]^+$ observed as precursors of m/z 256.1 (Figure 6b) was also quite similar to the acrolein-modified GPEtn species detected by precursor ion scanning for m/z 238.1 (Figure 6a). It appeared that acrolein readily adducted GPEtn species in HL-60 cells, but adduct formation did not seem to favor a particular subclass of GPEtn lipid (diacyl, ether, or plasmalogen) or which fatty acyl groups were esterified to the glycerol backbone.

HL-60 cells were then exposed to 0, 10, 30, 70, and 100 μ M acrolein for 30 min to determine if acrolein-modified GPEtn could be formed under conditions more relevant to acrolein concentrations found in either the lung of a smoker or acrolein produced in vivo. After the 30 min acrolein exposure, phospholipids from HL-60 cells were extracted, and the internal standard was added, reduced by sodium borohydride, and then injected onto the normal phase column with fraction collection (14–20 min) with a fraction of the effluent diverted to a mass spectrometer. The normal phase HPLC fractions were subsequently analyzed by reversed phase LC/MS/MS analysis. The elution of acrolein-modified GPEtn species from the reversed phase column was detected by precursor ion scanning for m/z 238.1 and m/z 256.1. The HMDP–lipid species (Figure 7a) and HMHP–lipid species (Figure 7b) were detected and quantitated using the added internal standard in HL-60 cells at concentrations of acrolein as low as 10 μ M acrolein. A trypan blue exclusion assay was used to determine if HL-60 cell membranes were intact after 30 min of treatment with 10, 30, 70, and 100 μ M acrolein. The HL-60 cell membranes were determined to be nonporous at the highest dose of acrolein (100 μ M); therefore, adduct formation of GPEtn lipids occurred in intact, live HL-60 cells. This trypan blue assay was simply used to indicate intact cells rather than address the mechanism of acrolein toxicity. In fact, delayed cell death induced by acrolein at those doses employed in these studies may be possible.

The time-dependent concentration of free acrolein during exposure of free acrolein during exposure to HL-60 cells was monitored using a 2,4-DNPH assay with UV detection of the acrolein hydrazone at 372 nm at doses of 10, 30, 70, and 100 μ M acrolein and at incubation times of 0, 3, 10, and 30 min. This 2,4-DNPH assay revealed a very rapid decrease in the concentration of free acrolein present in HL-60 cells treated with 10 and 30 μ M acrolein. In fact, most of the free acrolein present at these concentrations disappeared by the 3 min time point. The 2,4-DNPH assay did show a decrease in the amount of free acrolein present in the system after treatment of HL-60 cells with 70 and 100 μ M acrolein, but there was still measurable free acrolein present at incubation times of 30 min. Therefore, at the incubation time of 30 min used in the experiments above reporting acrolein-modified GPEtn, there was little free acrolein present in the 10 and 30 μ M acrolein-treated HL-60 cells, but there was still 30 and 52 μ M acrolein remaining in the 70 and 100 μ M-treated HL-60 cells, respectively. It should be emphasized that even though free acrolein was present in the system at 70 and 100 μ M, the excess acrolein was removed from the cells when they were pelleted and prior to extraction.

Discussion

Acrolein is a highly reactive α,β -unsaturated aldehyde that is a widespread environmental pollutant formed by the combustion of fossil fuels (1) and is one of the most abundant aldehyde compounds present in cigarette smoke (2). In addition, acrolein is formed endogenously during lipid peroxidation (11), neutrophil myeloperoxidase-catalyzed amino acid oxidation (25), and as a metabolism product of the anticancer drug cyclophosphamide (26). The toxicity of acrolein has been examined in many different cellular systems. For example, acrolein induces apoptosis in keratinocytes (27), neutrophils (7), neurons (28), CHO cells (29), and A549 cells (30) and induces necrosis in PC12 cells (31). In certain cells, acrolein effects biochemical processes including transcription factor activation (5), production or inhibition of inflammatory cytokines (32,33), and respiratory burst activation (34). The exact mechanism of these cellular effects of acrolein has not been established, but there is evidence that the alteration of these biochemical processes is due to the high chemical reactivity of acrolein with amino groups on proteins and possibly other endogenous nucleophilic compounds.

The molecular basis for the cellular toxicity of acrolein has been thought to be a result of covalent reactions with cellular nucleophiles (8). Acrolein readily reacts with 2'-deoxyguanosine and forms the 1,N²-propano-2'-deoxyguanosine adduct (35–37) with native DNA. This 1-N²-propano-2'-deoxyguanosine adduct was detected in DNA isolated from human and animal tissues (38,39), and the levels of this adduct were found to be significantly higher in the oral tissues of cigarette smokers as compared to nonsmokers (40). Such acrolein adducts of 2'-deoxyguanosine were found to be mutagenic in vitro and in vivo (41,42). Acrolein readily reacts with lysine side chains of proteins and forms quite stable adducts. The acrolein-lysine adducts identified include N^ε-(3-formyl-3,4-dehydropiperidine)lysine (9) and N^ε-(3-methylpyridinium)lysine (10). Using a specific antibody, acrolein-protein adducts have been detected immunohistochemically in Alzheimer's diseased brains (12), in the spinal cord after traumatic injury (43), and in atherosclerotic lesions (44). While covalent modifications of DNA and proteins with acrolein have been the focus of many investigations, the reaction of acrolein with glycerophosphoethanolamine lipids, another major cellular nucleophile, has not been reported.

The formation of the major products formed upon the reaction of acrolein with GPEtn lipids can be explained (Scheme 1). The primary amine group present on the GPEtn lipid undergoes 1,4-Michael addition with acrolein to form a secondary amine that contains an aldehyde group. This secondary amine can undergo another 1,4-Michael addition with acrolein, which results in the formation of a tertiary amine that contains two aldehyde groups (**III**). This particular

structure can then undergo aldol condensation to form FHP lipid (**IV**), which is a piperidine-derived product that contains one aldehyde group. This was the major product formed in the reaction of GPEtn with acrolein, and the $[M + H]^+$ of this product was present in the positive ion mass spectrum as a methanol hemiacetal. To confirm that the major product was product **IV** and not the isobaric product **III** in Scheme 1, MOX and BSTFA derivatizations and sodium borohydride reduction were performed. Both the derivatization procedures and the reduction indicated that the major product contained one aldehyde group and one hydroxyl group. This led to the conclusion that FHP lipid is the major product formed. To remove the possibility of hemiacetal formation during electrospray ionization and to remove the reactive aldehyde group, sodium borohydride reduction was performed as early as possible in the extraction process during our biological studies. Additionally, it is possible for the aldol condensation product that resulted from two 1,4-Michael additions of acrolein to undergo dehydration to form 1,2-diradyl-*sn*-glycero-3-phosphoethanol-(3-formyl-3,4-dehydro)piperidine (FDP) (**V**). This particular product was only observed as the sodium borohydride-reduced analogue.

To test whether acrolein would form adducts with endogenous aminophospholipids in a phospholipid bilayer, HL-60 cells were exposed to acrolein at various concentrations for 30 min. The phospholipid adducts were detected using a specific mass spectral assay following reduction of the aldehyde group present on the initial covalent adduct by sodium borohydride. The $[M + H]^+$ of the reduced products of both FHP lipid and FDP lipid molecular species were observed as abundant positive ions following electrospray ionization. Collisional activation of the $[M + H]^+$ from HMHP lipid and HMDP lipid molecular species revealed product ions at m/z 256.1 and 238.1, respectively, that contained head group information that was unique to these acrolein-modified GPEtn lipids. Therefore, precursor ion scanning for m/z 256.1 and 238.1 was used to selectively detect HMHP and HMDP molecular species present after exposure of HL-60 cells to acrolein. Using the selective mass spectral assay, the formation of acrolein GPEtn adducts was observed after 30 min when treating HL-60 cells with as low as 10 μ M acrolein, and the absolute quantity of adducts increased with acrolein concentration. A trypan blue exclusion assay indicated that HL-60 cell membranes were nonporous upon treatment with acrolein (10, 30, 70, and 100 μ M) for 30 min, which suggested that the GPEtn-acrolein adducts were formed in intact cells. In addition, it was ensured that these acrolein GPEtn adducts were not formed during sample preparation for mass spectral analysis by pelleting the cells and removing the supernatant with any excess acrolein before Bligh and Dyer extraction.

The modification of the primary amine group on GPEtn lipids by acrolein might have biological significance from several standpoints. The membrane fluidity could be affected by acrolein-modified GPEtn lipids, which could ultimately result in an alteration of membrane structure and function. In a previous study, it was found that accumulation of malondialdehyde disturbs the organization of aminophospholipids in the erythrocyte membrane, and this disruption was thought to be caused by the ability of MDA to cross-link through Schiff base formation between aminophospholipids (45). There is also a possibility that acrolein GPEtn adducts could alter the enzymatic activity of various phospholipase enzymes that act on cellular phospholipids. For example, Guichardant and co-workers have found that the 1,4-Michael adduct of 4-hydroxy-2 *E*-nonenal was a poor substrate for pancreatic phospholipase A₂ and of cabbage phospholipase D (46). Therefore, it is possible that these acrolein GPEtn adducts could modulate signaling events within a cell. Additionally, the acrolein GPEtn adducts could be important in oxidative injury type disease states, such as diabetes (47), atherosclerosis (17), and Alzheimer's disease, or most likely in cigarette smoke-related diseases of the lung because of the high concentrations in main stream smoke. Furthermore, Zieseniss et al. found that Schiff base aldehyde-GPEtn adducts in oxidized low-density lipoproteins were responsible for eliciting a pronounced prothrombic response by increasing the activity of platelet prothrombinase complex (18). Finally, because the plasma membrane is the first line of defense

for cells, it is possible that GPEtn lipids serve as the first barrier to acrolein and protect critical cellular nucleophiles from this reactive oxygen species.

In summary, the reaction of acrolein with glycerophosphoethanolamine lipids is quite facile, and covalent adducts of acrolein with GPEtn lipids have been characterized using electrospray tandem mass spectrometry. The major product formed involved the addition of two acrolein molecules to the primary amino group of GPEtn lipids with subsequent aldol condensation to form FHP lipids. Upon sodium borohydride reduction, the acrolein GPEtn adducts could be selectively detected using electrospray tandem mass spectrometry by employing precursor ion scanning for m/z 256.1 and 238.1. These precursor ion scans were successfully used to detect acrolein GPEtn adducts formed upon treatment of HL-60 cells with relatively low concentrations of acrolein. While the biological role for these acrolein GPEtn adducts has not been established, these structural characterization studies and analysis of products formed in the intact cell reported herein reveal for the first time the potential role of aminophospholipids in cellular membranes as targets for this highly toxic and electrophilic reactive oxygen species.

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Figure 1.

(a) Positive ion electrospray mass spectrum of 18:1a/18:1-GPEtn. The ion at m/z 744.5 is the [M + H]⁺ of 18:1a/18:1-GPEtn. The ions at m/z 766.5 and 788.5 correspond to the sodium and disodium adduct of m/z 744.5. (b) Positive ion electrospray mass spectrum of the products formed upon the reaction of 18:1a/18:1-GPEtn with 3 mM acrolein. The ions at m/z 856.7 and 888.6 correspond to the [M + H]⁺ of 18:1a/18:1-FHP and the methanol hemiacetal of this compound, respectively. The ions at m/z 910.6 and 932.6 correspond to the sodium and disodium adduct of m/z 888.6. The inset is the structure of the [M + H]⁺ at m/z 888.6, which is the methanol hemiacetal of 18:1a/18:1-FHP.

Figure 2.

Positive ion electrospray mass spectra of the products formed upon the reaction of 18:1a/18:1-GPEtn with (a) 10, (b) 100, and (c) 500 μM acrolein. The ion at m/z 888.6 corresponds to the $[\text{M} + \text{H}]^+$ of the methanol hemiacetal of 18:1a/18:1-FHP. The ions at m/z 910.6 and 932.6 correspond to the sodium and disodium adduct of m/z 888.6.

Figure 3.

(a) Positive ion electrospray mass spectrum of 18:1a/18:1-FHP after MOX derivatization. The major $[M + H]^+$ present in the spectrum at m/z 885.6 is the MOX derivative of 18:1a/18:1-FHP, and the ions at m/z 907.6 and 929.6 are the sodium and disodium adducts of this compound. The inset is the structure of the $[M + H]^+$ at m/z 885.6, which is the MOX derivative of 18:1a/18:1-FHP. (b) Positive ion electrospray mass spectrum of 18:1a/18:1-FHP after MOX/BSTFA derivatization. The $[M + H]^+$ at m/z 957.6 is the MOX/BSTFA derivative of 18:1a/18:1-FHP. The ions at m/z 979.6 and 1001.6 correspond to the sodium and disodium adducts of this compound. The inset is the structure of the $[M + H]^+$ at m/z 957.6, which is the MOX/BSTFA derivative of 18:1a/18:1-FHP.

Figure 4.

(a) Positive ion electrospray mass spectrum of 18:1a/18:1-FHP after reduction with sodium borohydride. The inset is the structure of the $[M + H]^+$ at m/z 858.6, which is 18:1a/18:1-HMHP. (b) CID of the $[M + H]^+$ of 18:1a/18:1-HMHP at m/z 858.6 at a collision energy of 35 V. The origins of the ions that resulted from collisional activation are indicated in the structure of this product. (c) CID of the $[M + H]^+$ of 18:1a/18:1-HMDP at m/z 840.6 at a collision energy of 35 V. The origins of the product ions are indicated in the structure of this product.

Figure 5.

(a) Normal phase HPLC separation of the different phospholipid classes extracted by a modified Bligh–Dyer from the sodium borohydride-reduced control and 500 μ M acrolein-treated HL-60 cells as described in the Experimental Procedures. The phospholipids were detected using a positive ion Q1 scan. The elution of each phospholipid class is indicated by the abbreviations GPEtn (glycerophosphoethanolamine), GPSer (glycerophosphoserine), and GPCCho (glycerophosphocholine) lipids. (b) Positive ion electrospray mass spectrum of GPEtn lipids present in HL-60 cells. The ions that are labeled in this figure are identified in Table 1. (c) Positive ion electrospray mass spectrum of acrolein-modified GPEtn species present in HL-60 cells after exposure to 500 μ M acrolein.

Figure 6.

(a) Precursors of m/z 238.1 scan and (b) precursors of m/z 256.1 scan of acrolein-modified GPEtn molecular species formed after incubation of HL-60 cells with 500 μ M acrolein that eluted from the normal phase column between 14–20 min. The ions that are labeled in this figure are identified in Table 1.

Figure 7.

Dose response of the detection of (a) HMDP lipids using a precursor of m/z 238.1 scan and (b) HMHP lipids using a precursors of m/z 256.1 scan after exposure of HL-60 cells to acrolein (0, 10, 30, 70, and 100 μ M acrolein) for 30 min. This dose response was constructed from the reversed phase chromatograms by extracting the mass range of m/z 750–800 and m/z 770–820 from the precursors of m/z 238.1 and m/z 256.1 reversed phase chromatograms, respectively. These mass ranges corresponded to all of the HMDP–lipid and HMHP–lipid species present after exposure of HL-60 cells to acrolein (Figure 5a,b), and the area underneath these reversed phase chromatographic peaks was determined. Then, the internal standard (acrolein-modified 14:0a/14:0-GPEtn) was extracted at m/z 732.6 and m/z 750.6 from the precursors of m/z 238.1 and precursors of m/z 256.1 reversed phase chromatograms, and the area underneath these chromatographic peaks was determined. The product signal was divided by the corresponding internal standard signal to observe changes in acrolein-modified GPEtn species formed in HL-60 cells upon exposure to 0, 10, 30, 70, and 100 μ M acrolein.

Scheme 1.

Table 1
Summary of Nonmodified GPEtn and Acrolein-Modified GPEtn Molecular Species Found in HL-60 Cells^a

GPEtn species	GPEtn [M + H] ⁺	HMDP ^b [M + H] ⁺	HMHP ^c [M + H] ⁺
16:0p/18:1-GPEtn ^d	702.6	798.5	816.6
16:0a/18:1-GPEtn	718.7	814.7	832.6
16:0p/20:4-GPEtn	724.6	820.6	838.6
18:0a/18:1-GPEtn	746.7	842.7	860.6
18:0p/20:4-GPEtn	752.5	848.7	866.7
18:0a/20:4-GPEtn	768.7	864.7	882.6
18:2p/22:4-GPEtn	776.7	872.6	890.7
18:2a/22:4-GPEtn	792.6	888.6	906.6

^aThese phospholipids were identified by performing negative ion CID on the corresponding [M – H][–], which provides information about the fatty acids esterified to the glycerol backbone.

^bHMDP is used as an abbreviation for (3-hydroxymethyl-3,4-dehydro)piperidine lipids.

^cHMHP is used as an abbreviation for (3-hydroxymethyl-4-hydroxy)piperidine lipids.

^dAbbreviations for lipids are described in footnote 2.